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A rapid method for detection and identification of flaviviruses by polymerase chain reaction and nucleic acid hybridization

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Summary. A polymerase chain reaction (PCR) technique was developed and evaluated for the detection of flaviviruses. A set of sense and antisense oligomeric DNA primers were constructed from nucleotide sequences of the conserved region of the genome of several different flaviviruses. Virus specific complementary DNA (cDNA) was prepared by reverse transcription of total RNA extracted from infected cell cultures. Amplified cDNA was identified by nucleic acid hybridization with specific oligomeric internal probes. Various conditions, such as number of cycles and annealing temperature were examined to optimize the detection of viral RNAs from infected cell cultures. Slot blot hybridization with a radioactive probe was used to evaluate the sensitivity of PCR amplification. The PCR amplified RNA sequences of dengue 2 (DEN-2), West Nile (WN), St. Louis encephalitis (SLE) and Kunjin (KUN) virus and detected 0.1 to 1 pg of viral RNA. Japanese encephalitis (JE), Yellow Fever virus (YF), DEN-1, 3, and 4 viruses were not amplified. The more frequent occurrence of mismatches in the 3' primer binding site may explain the failure to amplify cDNA of these viruses.

Introduction

Several of the viruses among the family *Flaviviridae* such as Yellow fever (YF), Dengue (DEN), Japanese encephalitis (JE), St. Louis encephalitis (SLE), Kunjin (KUN), West Nile (WN) and Tick born encephalitis are important causes of human disease [18]. The laboratory diagnosis of flaviviral infections currently depends upon the demonstration of virus specific antibodies in patient sera or the isolation and identification of these viruses followed by serologic typ-

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ing [8, 10, 13]. These methods usually take two or more weeks, thus delaying laboratory diagnosis until long after resolution of the patient's illness. The polymerase chain reaction (PCR) has been shown to be a rapid and sensitive method for diagnosing viral diseases [4, 11, 14]. Recently, Eldadah and others [5] adapted the RT-PCR method for the detection of flaviviruses. In the present study we describe the sensitivity and specificity of a unique set of primers which can be used for amplification of specific regions of the genomes of DEN-2, WN, SLE and KUN viruses.

Materials and methods

Viruses

The cultivation of dengue viruses in C6/36 (*Aedes albopictus*) cells and titration in LLC-MK₂ (rhesus monkey kidney) cells have been described previously [9]. Dengue virus strains used in this study included DEN-1 (Hawaii), DEN-2 (New Guinea strain), DEN-3 (Thailand, CH53489), and DEN-4 (Dominica, 814669). YF (17D) and KUN viruses were maintained as low passage C6/36 cells culture stocks. JE (Nakayama) and SLE viruses were provided by Dr. E. A. Henchal of Walter Reed Army Institute of Research. The WN virus stock was provided by Dr. R. Shope of Yale University. A rhinovirus stock obtained from ATCC was used as a nonflavivirus control. Viral preparations used for specificity testing contained at least 10⁵ plaque forming units/ml.

Preparation of RNA

Viral RNA was extracted with a mixture of guanidine isothiocyanate-phenol-chloroform by modified methods described previously [2]. 100 ml of each virus suspension was mixed with solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol). Sodium acetate, pH 4.0, was added to yield a final concentration of 0.2 M, and the viral RNA was extracted with an equal volume of water saturated phenol and one fifth volume of chloroform:isoamyl alcohol (49:1). The sample was then mixed vigorously for 10 sec and cooled on ice for 15 min and centrifuged at 10000 rpm in a sorvall 33-34 rotor (Dupont, Wilmington, DE) for 20 min at 4 °C. The top aqueous layer containing viral RNA was precipitated with an equal volume of ice cold isopropyl alcohol for one h at -20 °C. The precipitated RNA was then centrifuged and the pellet was washed with 70% ethanol. After centrifugation, the pellet was dried using a Savant (Farmingdale, NY) Speed Vac concentrator.

Synthetic DNA oligomeric probes and primers

Sense and antisense primers were designed with the use of a computer-assisted analysis of the genomic RNA of several different flaviviruses which have been fully sequenced. The primer sequences chosen were from conserved regions of the envelope protein except for a few internal mismatches. Specific probes were selected for each virus from unique base sequences within the PCR amplified regions. Reagent grade synthetic DNA oligomeric primers and probes (Table 1) were produced by Synthecell (Rockville). Table 2 shows the comparison of viral sequences with the flaviviral primers, and the length of PCR products amplified by the primers for each virus. For internal mismatches, we inserted the neutral nucleoside inosine at two places.

Table 1. Sequences of oligomer DNA primers and probes for PCR amplification of viral RNA

Oligomer ^a	Number ^b	Sequence ^c
Primers		
Flavi 1 (–)		CCTCCAAIGGAICCAAAATCCCA
Flavi 2 (+)		GGATGGGGIAATGGITGTGG
Probes		
DEN-2	1419	AGTCAAGATAACACCACAGAGC
WN	1399	GGCCCGACGACCGTTGAATCTC
SLE	1395	GGTTCAACGGACTCTACGTCAC
KUN	1378	GGACCAACTACCGTGGAATCGC

^a Oligomers were either of genomic (+) or antigenomic (–) sense

^b Number corresponds to nucleotide base sequence. DEN-2 specific (7), WN specific (1), SLE specific (23), and KUN specific (3)

^c Sequences are presented in the 5' to 3' direction

Table 2. Comparison of viral sequences with flaviviral primers

Virus strain	Viral sequences (5'–3')	Number ^j	Predicted size of PCR product (bp)
DEN 1 ^a	1. CCTCCTATAGAACCGAAGTCCCA	2165	
	2. GGCTGGGGCAATGGTTGTGG	1205	983
DEN 2 ^b	1. CCTCCCAGAGATCCAAAATCCCA	2194	
	2. GGATGGGGAAATGGATGTGG	1234	986
DEN 3 ^c	1. CCACCCACTGATCCAAAGTCCCA	2194	
	2. GGCTGGGGAAACGGTTGTGG	1234	986
DEN 4 ^d	1. CCACCAACGGAACCAAAATCCCA	2194	
	2. GGGTGGGGCAATGGCTGTGG	1234	986
WN ^e	1. CCTCCAACTGATCCAAAATCCCA	2230	
	2. GGATGGGGAAATGGCTGCGG	1264	986
SLE ^f	1. CCTCCAAATAGATCCAAAGTCCCA	2238	
	2. GGATGGGGTAACGGATGTGG	1260	1001
KUN ^g	1. CCTCCAACCGATCCAAAGTCCCA	2221	
	2. GGTTGGGGCAATGGGTGCGG	1243	1001
JEV ^h	1. CCTCCAAATAGAGCCAAAGTCCCA	1939	
	2. GGGTGGGGCAACGGATGTGG	964	998
TT ⁱ	1. CCTCCAGCGGAGCTGAAATCCCA	2225	
	2. GGCTGGGGCAATGGCTGTGG	1271	976

Mismatches in viral sequences from Flavi 1 (+) and Flavi 2 (+) primers are in italics

^a Mason et al. [17]; ^b Hahn et al. [7]; ^c Osatomi et al. [22]; ^d Mackow et al. [15]; ^e Castle et al. [1]; ^f Trent et al. [23]; ^g Coia et al. [3]; ^h McAda et al. [19]; ⁱ Rice et al. [21]. ^j Number indicates the map site at which 5' end of Flavi primers hybridize on each viral strain genome

Reverse transcriptase-polymerase chain reaction assay (RT-PCR)

Virus specific cDNA was amplified by RT-PCR using the flaviviral primers. The dry RNA pellets were resuspended in 10 μ l of sterile distilled water, and 2 μ l of primer 1 (11.8 pmoles) was added. The RNA suspension was heated at 68 °C for 3 min, cooled on ice and added to 18 μ l of reaction mixture containing 50 mM Tris pH 8.8, 200 mM ammonium sulphate, 25 mM MgCl₂, 10 mM dithiothreitol, 0.1% triton X-100, 100 mg/ml BSA, 275 mM deoxynucleotide triphosphate, 25 units RNAsin (human placental ribonuclease inhibitor; Promega, Madison, WI) and 10 units of reverse transcriptase in the total volume 30 μ l. The mixture was incubated at 42 °C for 40 min and cooled on ice. Polymerase chain reaction amplification of cDNA was performed by adjusting the mixture to 50 mM Tris pH 8.8, 200 mM ammonium sulphate, 25 mM MgCl₂, 200 mM deoxynucleotide triphosphate, 0.3 mM primer 1 and 0.3 mM primer 2 and 0.025 units/ml of Amplitaq DNA polymerase (Perkin-Elmer Cetus Corporation) in a total volume of 100 μ l. To deduce the optimal reaction conditions, two magnesium concentrations (1.5 mM and 2.5 mM) and three different numbers of total cycles (25, 30 and 35) were investigated. Following PCR the samples were cooled to 4 °C. A 10 μ l aliquot of each sample was electrophoresed on a 4–20% NOVEX TBE polyacrylamide gradient gel (Encinitas) and stained with ethidium bromide using standard method [16]. Stained gels were photographed on a light box using Polaroid type 667 film. Slot blot hybridization of the PCR samples was done on Hybond-N membrane.

Preparation of labelled oligomeric probes

Oligomeric DNA probes specific for each virus were 5'-end labeled with ³²P using T-4 DNA Kinase (Boehringer Mannheim, Indianapolis, IN) and partially purified using Sephadex G-25 column chromatography according to standard method [16]. Radiolabeled synthetic probes were diluted to 10⁶ cpm/ml in hybridization buffer before use.

Nucleic acid hybridization

A 10 μ l aliquot of each amplified DNA sample was treated with 1 μ l of 3 M sodium hydroxide and incubated for 30 min at 70 °C. Samples were neutralized with two volumes of 2 M ammonium acetate, vortexed and cooled on ice. An aliquot of each sample was slotted onto Hybond N-nylon membrane using a Stratalinker UV box (Stratagene) according to the manufactures instructions. Membranes were treated with an excess volume of prehybridization buffer (6 \times SSC [1 \times SSC = 0.15 M sodium chloride, 0.015 M sodium citrate], 5 \times Denhardt's solution [1 \times Denhardt's = 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin], 0.5% sodium dodecyl sulfate, 0.05% sodium pyrophosphate and 0.1 mg/ml sheared salmon sperm DNA) for 2 h at 37 °C in a heat sealed bag. The membranes were then hybridized with ³²P labelled oligomeric probes overnight at 37 °C in hybridization buffer (6 \times SSC, 1 \times Denhardt's solution, 0.5% sheared salmon sperm DNA, 0.15% sodium pyrophosphate). Membranes were washed twice with an excess of 2 \times SSC, 1% sodium dodecyl sulfate for 15 min at ambient temperature, twice with 0.2 \times SSC and 0.1% sodium dodecyl sulfate for 15 min at 37 °C, and twice with 0.2 \times SSC and 0.1% sodium dodecyl sulfate for 15 min at 42 °C. Washed membranes hybridized with ³²P labelled probe were air dried and exposed to Kodak X-OMAT XAR-5 film (Eastman Kodak) and Cronex lightening plus intensifying screen (Dupont).

Clinical specimens

Human serum samples were obtained from patients with clinically characterized dengue-2 infection were tested by the RT-PCR assay. These samples had previously been shown

to contain DEN-2 viruses by cell culture isolation and identification of virus serotype by indirect immunofluorescence assay (IFA) with dengue virus type-specific monoclonal antibodies.

Results

The specificity of the flaviviral primers was evaluated by RT-PCR employing flaviviral, nonflaviviral infected and uninfected negative control cell culture supernatants. The combination of 35 cycles at 1.5 mM $MgCl_2$ and 42 °C annealing provided the highest level of sensitivity and specificity. The RT-PCR amplified the RNA of DEN-2, WN, SLE, and KUN viruses, but failed to amplify JE, YF, DEN-1, 3, and 4. The size of the amplified DNA was consistent with the predicted for the target region in the viral genomes (between 986–1001 bp) (Table 2). No PCR product was obtained with flaviviral primers used for rhinovirus and uninfected cell culture supernatant. The PCR products demonstrated by ethidium bromide staining are shown in Fig. 1.

The flaviviruses were identified by nucleic acid hybridization of specific oligomeric DNA probes with amplified cDNA. Slot blot hybridization was chosen as a method of evaluation because it allowed for simultaneous identifi-

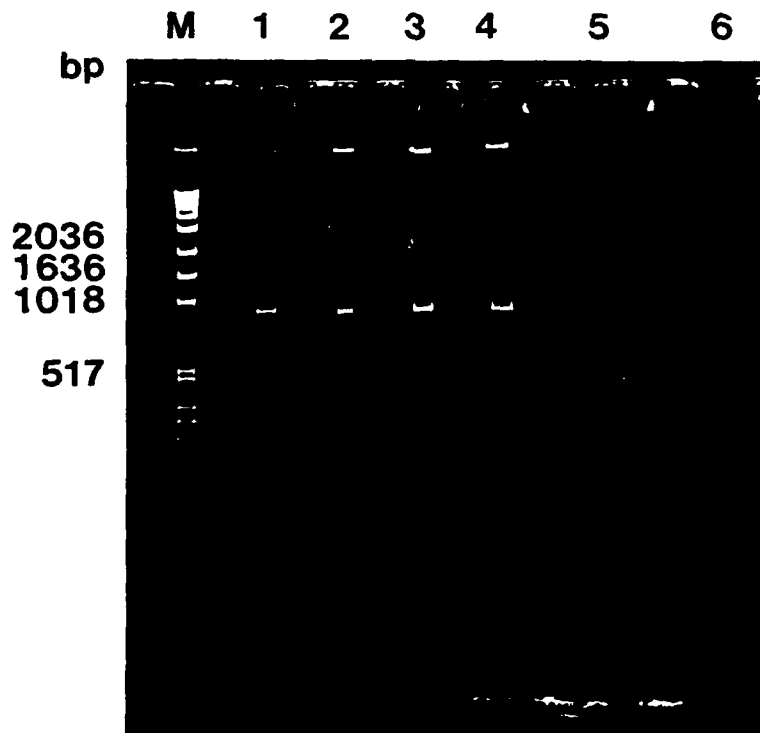


Fig. 1. Analysis of polymerase chain reaction amplification products separated by electrophoresis on a 20% polyacrylamide gradient gel and stained with ethidium bromide. M 1 Kb: DNA ladder standard. 1 DEN-2; 2 WN; 3 SLE; 4 KUN; 5 Rhinovirus; 6 Uninfected cell culture supernatant

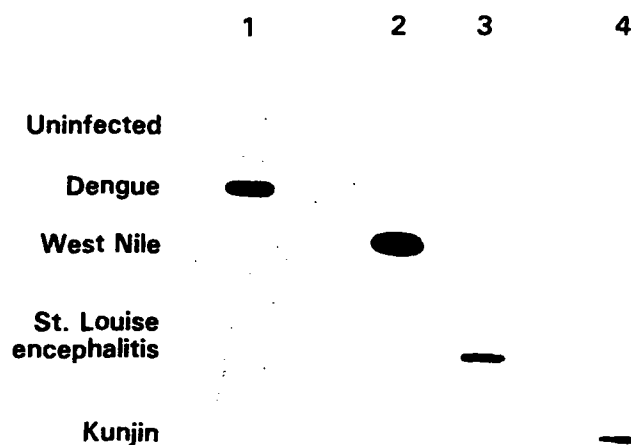


Fig. 2. Slot blot hybridization of the PCR products resulting from the enzymatic amplification of RNA extracted from uninfected, DEN-2, WN, SLE, and KUN infected cell cultures. 1 DEN-2; 2 WN; 3 SLE, and 4 KUN specific probes



Fig. 3. Sensitivity of the reverse transcriptase-polymerase chain reaction assay to detect the viruses. PCR products from different amount of RNA from uninfected, DEN-2, WN, SLE, and KUN infected cell cultures were slot blotted and hybridized with radiolabeled probes. 1 DEN-2; 2 WN; 3 SLE; and 4 KUN

cation using different synthetic probes. Amplified cDNA from uninfected, DEN-2, WN, SLE and KUN were each hybridized against specific oligomeric DNA probes (Table 1). Each oligomeric probe reacted only with the homologous virus (Fig. 2).

In order to determine the sensitivity of the flaviviral primers, titration experiments were done with DEN-2, WN, SLE and KUN viruses. RNA was extracted from DEN-2, WN, SLE, and KUN infected cell culture supernatants and quantitated by spectrophotometry. Three 10 fold dilutions of each RNA sample was made in distilled water. Ten μ l of each dilution (1 pg, 0.1 pg, and 0.01 pg RNA) was used for RT-PCR amplification. Following amplification, 1 pg of RNA from DEN-2 and WN and 0.1 pg of RNA from SLE and KUN was detected directly by ethidium bromide staining. The amplified product from

Table 3. Comparison of RT-PCR/slot blot hybridization and virus isolation for the identification of DEN-2 viruses from human serum

Method	No. of serum samples of DEN-2 virus type
Virus isolation	13
RT-PCR and slot blot hybridization	12

each virus only reacted with the homologous oligomeric probe by slot blot hybridization. Faint bands were detected with the amplified products from 0.1 pg RNA of all four viruses by nucleic acid hybridization (Fig. 3).

Sera from thirteen human viremic patients and three virus negative control sera were tested by RT-PCR assay. Amplified DNA was first characterized by gel electrophoresis and staining by ethidium bromide, followed by slot blot nucleic acid hybridization. Table 3 summarizes the results comparing identification by the RT-PCR assay with identification by virus isolation in cell culture and subsequent typing by the IFA with type-specific monoclonal antibodies. In all but one instance, DEN-2 viruses were correctly detected and typed by the RT-PCR assay, compared with virus isolation. Negative samples were negative, and one sample containing DEN-2 virus was not found positive by the RT-PCR method.

Discussion

All known flaviviruses share short conserved sequences. The gene which codes for the envelope protein exhibits sequences with minimal variations among the several different flaviviruses. This conservation allowed the selection of a pair of flaviviral primers which could be used for amplification of the envelope gene from several different flaviviruses even though they are not perfectly complementary within the short 20–23 nucleotides length of the primers.

Previous investigators have described the PCR assay for St. Louis encephalitis (SLE), Japanese encephalitis (JE), yellow fever (YF), dengue 2 (DEN-2) and dengue-4 using different sets of primers for each virus [5]. Here, we evaluated RT/PCR amplification of RNA from four different viruses within the flavivirus group using a single set of flaviviral primers followed by nucleic acid hybridization with radiolabeled synthetic oligomeric probes. Twenty-three base antisense and twenty base sense primers (Table 2) amplified the predicted size of fragments from DEN-2, WN, SLE, and KUN. An internal 22 base oligonucleotide from each member served as a probe. Using these primers, a protocol was developed that detected as little as 1.0–0.1 pg of RNA from DEN-2, WN, SLE and KUN after ethidium bromide staining. The more occurrence of mismatches and or mismatches closer to 3' prime binding site may explain the lesser efficiency of amplification with YF, JE, DEN-1, 3, and 4 viruses. Important considerations

in developing a PCR protocol are optimizing the number of cycles, annealing temperature and magnesium concentration. At an annealing temperature greater than 42°C there was no amplification, and at lower temperatures, there were additional nonspecific bands.

Since a single set of flaviviral primers was designed to amplify different viruses it was necessary to identify each virus type after amplification. The specificity of the amplified fragment was confirmed by an internal sequence specific oligonucleotide probe. The use of radiolabeled oligonucleotide probe allowed for an additional level of specificity (96–100%) for detection of PCR products.

Because of the high degree of sensitivity and specificity, the PCR method described here has a potential application for the routine diagnosis of flaviviral infections. The diagnostic usefulness of our assay is demonstrated by the analysis of human serum samples containing dengue virus. Another decided advantage of PCR is speed; the entire procedure can be completed in 8 h and multiple samples can be tested simultaneously with minimal technical effort. However, the current PCR assay is limited in its usefulness because it only amplified 4 of the 9 flavivirus recognized. This limitation may be overcome by using primer pairs with greater homology to the other flaviviruses not amplified by Flavi-1 and Flavi-2 or by constructing degenerate primers.

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